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TECHNICAL MANUSCRIPT 394

STUDIES ON ANTHRAX TOXIN PRODUCED IN VIVO

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Ralph E. Lincoln

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DEPARTMENT OF THE ARMY
Fort Detrick
Frederick, Maryland

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In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Academy of Sciences-National Research Council.

ABSTRACT

Specific anthrax antigens were demonstrated in the blood of animals dying from anthrax. These antigens appeared in the blood when organisms were first detected and their concentration continued to increase as the concentration of organisms increased. These antigens did not elicit a strong antibody response. In some respects, the toxin produced in vivo is different from that produced in vitro (may kill more rapidly, harder to detect lethal factor). The in vivo toxin also exists as an aggregate whose biological and serological activity depends upon its particular composition and/or configuration.

I. INTRODUCTION

In addition to the identification and purification of in vivo - produced toxin,[1-3] its ability to act as an aggressin has also been studied (Smith et al.[4]). However, aside from the studies by Smith and colleagues, especially those of Tempest and Smith[5] on toxin production and those of Sargeant, Stanley, and Smith[6] on the serological relationship between purified preparations from toxin produced in vivo and in vitro, little is known of the synthesis of this molecule or the extent of its differences from the toxin produced in vitro.

This paper presents the results of our studies on the kinetics of toxin production, the formation of specific antiserum, differences between toxin produced in vivo and in vitro, and studies on the dissociation of the toxin molecule.

II. MATERIALS AND METHODS

A. CULTURE CONDITIONS

Spore stocks were prepared by growing Bacillus anthracis strains V1b, 116, 770, and Sterne in N-Z-Amine, Type A medium for 24 hours. Spores, collected by centrifugation, were washed with water until negative for catalase and free of sporangia by light microscopic examination. They were heat-shocked at 60 C for 30 minutes and then stored in the presence of 0.1% phenol at 4 C until used.

Vegetative cells were prepared by growing in casein acid digest medium. Fifty ml of medium in a 250-ml Erlenmeyer flask were inoculated and placed at 37 C on a reciprocating shaker (60 two-inch strokes per minute). Subcultures were prepared by inoculating 0.5 ml into fresh medium; populations of 1×10^9 cells per ml were obtained in 24 hours. This subculture was used within 2 hours.

B. ANTHRAX TOXIN AND ITS COMPONENTS

The method of Fish, Mahlandt, and Lincoln[7] was used to prepare whole toxin; protective antigen (PA), after final filtration; edema factor (EF), 35-fold purified; and lethal factor (LF), 35-fold purified.

C. ASSAY CONDITIONS

Serological (antigenic) activity was determined by the Ouchterlony method[8] using azocarmine.[7] The antiserum (DH-1-6C) had been prepared by repeated injections of spores of the Sterne strain of B. anthracis into a horse. Rat lethality was measured by the method of Haines, Klein, and Lincoln[9] and guinea pig skin edema by the method of Beall et al.[10]

D. OTHER PROCEDURES

Animals were immunized by five injections of protective antigen (PA5), a live vaccine (LV), or a combination of these (PA5 plus LV). The procedure used was that described by Klein et al.[11] Cellulose acetate electrophoresis[12] was used to examine the sera of monkeys that died from anthrax.

III. RESULTS

A. KINETICS OF IN VIVO TOXIN PRODUCTION

Guinea pigs (Hartley strain, 300 to 400 g) were injected intraperitoneally (IP) with 1 ml of a fresh culture containing 8×10^8 to 16×10^8 cells per ml. The guinea pigs died in 18 to 20 hours following challenge with V1b or 116 strains and in 26 to 42 hours following challenge with 770 or Sterne strains. The fluid in the thoracic and peritoneal cavities was collected and Ouchterlony plates were prepared. Figure 1 shows that every sample contained antigen, as demonstrated by lines of precipitation against antiserum. In some of the samples, two lines of precipitation can be visualized; in others, only one line is visible.

In a second experiment, several guinea pigs were injected IP with 1 ml of 1×10^8 V1b spores. Individual guinea pigs were bled at 2- to 3-hour intervals and their sera were assayed for the presence of antigen. The first trace of antigen was found 6 hours after challenge. Several negative samples were then found, but by 17 to 22 hours a single, strong line of precipitation was observed. Between 23 and 28 hours two lines of precipitation were clearly visible and at the end of the experiment (30 hours) a third line was also visible.

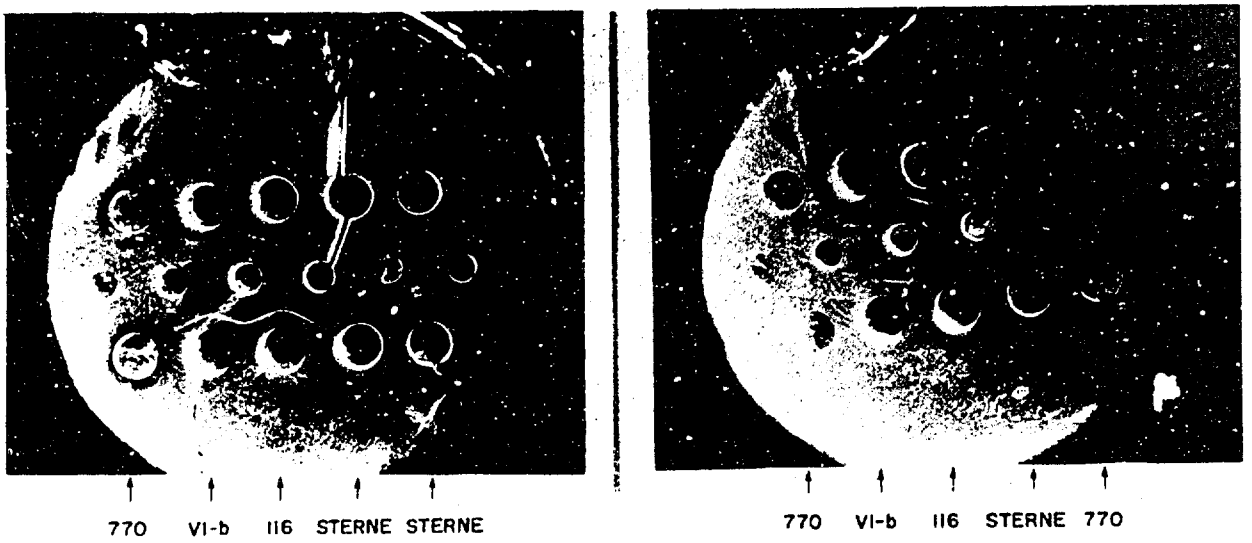

Figure 1. Demonstration of the Presence of Antigens in the Thoracic (left) and Peritoneal (right) Fluids of Guinea Pigs after Challenge with Different Strains of Anthrax.

In a parallel set of experiments, five rhesus monkeys (4 to 5 pounds) were challenged intramuscularly (IM) with 2×10^9 V1b spores. The results (Table 1), indicate the relationship between organism and toxin production in that the longer the animal survives, the higher the cell count and the more toxin present in the blood. Cellulose acetate electrophoresis revealed an identical pattern using the serum before challenge and at time of death. Two of the animals had serosanguinous exudate in the peritoneal and thoracic cavity that contained antigen by Ouchterlony analysis (two lines of precipitation at a dilution of 1:16) and by its ability to kill rats following intravenous (IV) injection (124 and 155 minutes).

TABLE 1. TIME COURSE OF ORGANISM AND TOXIN CONTENT OF THE SERUM AFTER SPORE CHALLENGE

Time to Death, hr	Terminal Viable Cell Count, org/ml	Rat Lethal Units/ml	Ouchterlony Titer
41	7.7×10^3	0	1:1
44½	2.1×10^3	0	1:1
45½	4.4×10^6	48	1:4
48	1.2×10^6	16	1:2
51½	2.5×10^8	307	1:16

In an experiment on the time course of toxin (antigen) production, the jugular vein of a monkey was cannulated and the monkey was challenged by an intradermal injection of 3×10^6 V1b spores. Five ml of blood were withdrawn at 4-hour intervals and assayed for the presence of toxin. The results are shown in Table 2. As soon as organisms could be demonstrated in the blood, it was possible to demonstrate toxin.

TABLE 2. TIME COURSE OF TOXIN FORMATION IN A MONKEY AFTER SPORE CHALLENGE

Time, hr	Viable Organisms/ml	Presence of Toxin[a]
0	0	0
4	0	0
8	1	0
12	0	0
16	6.6×10^3	+
20	2.3×10^6	+
22[b]	8.4×10^6	+

a. Toxin was detected by the Ouchterlony analysis.
b. Taken immediately after death of the animal..

B. FORMATION OF SPECIFIC ANTISERUM

Guinea pigs were immunized with whole toxin or PA by the PA5 protocol. Following injection with whole toxin, antiserum against LF, but not against PA, could be demonstrated. Injection with PA induced the formation of antiserum specific for PA. The titers of these sera were quite low (1:4) compared with those of the horse serum normally used (1:256).

When rabbits (New Zealand white, 2 to 4 pounds) were injected with whole toxin or its components by the PA5 protocol, no antibody could be demonstrated in the initial bleedings. However, if the protocol was repeated a month later, and then a month after that, a moderately titered (1:16) antiserum was produced. In contrast to the results obtained in guinea pigs, whole toxin stimulated the production of antibodies against all three components. PA and EF stimulated the production of antibodies to PA; LF caused the production of antibodies to PA and LF.

Rabbits were next injected IP with 1×10^5 or 1×10^9 spores of V1b, 770, 116, and Sterne strains weekly for the first 4 weeks. The following antibiotic treatment was employed: the rabbits were injected IM at 12, 24, and 36 hours with 40,000 units of crystalline penicillin per pound and 7.5 mg of aureomycin per pound, followed by IM injection at 48 and 72 hours with 10,000 units of procaine penicillin per pound.

Within 3 months, the serum from rabbits injected with V1b contained antibodies against all three of the toxin components isolated from the Sterne toxin. The serum from the rabbits that had received 770, 116, and Sterne contained antiserum against PA and LF but not against EF. The immunization was continued for another 3 months with no antibiotic therapy, and at that time the serum from the animals injected with V1b and Sterne contained antibodies against all three toxin components, but the serum from the rabbits injected with 770 contained antibodies only against EF. The rabbits were held for 6 months with no further treatment. Then they were challenged by IP injection of 2×10^9 V1b spores. The immunized animals survived but nonimmunized controls died in 73 and 88 hours. The prechallenge serum did not contain any visible antibody titer.

In similar experiments, three monkeys were individually immunized by the PA5, LV, or PA5 plus LV protocol. Their respective antibody titers against whole toxin were two lines of precipitation at a dilution of 1:1, one line of precipitation at a dilution of 1:16, and no lines of precipitation at a dilution of 1:1.

Eight monkeys were challenged with V1b spores and cured with penicillin. Upon rechallenge, none of the monkeys died. Their blood was collected and the serum titered against PA and LF. The sera had antibody titers of 1:2 to 1:16 and 1:1 to 1:8 respectively for the two components.

In another experiment, one monkey had been challenged with V1b and cured. The animal survived challenge with 1×10^7 V1b spores 4.5 years later. When challenged 1 week later with 1×10^9 spores the animal died. Examination of the terminal serum revealed an antigen titer of 1:4 and an antibody titer of 1:16.

C. DIFFERENCES BETWEEN TOXIN PRODUCED IN VIVO AND IN VITRO

Using in vitro - produced toxin, Haines et al.[9] and we* have never been able to kill rats in less than 54 to 56 minutes, regardless of the dose of toxin injected. However, on two occasions we were able to kill rats very rapidly with toxin produced in the monkey. The first monkey had been injected with 1×10^2 V1b spores and treated with 1×10^6 units of penicillin 72 and 86 hours later. The animal died 90 hours after challenge, and when 1.0 ml of its blood was injected into a rat, the rat died in 10 minutes. Rats receiving 1.0 ml of normal monkey blood showed no ill effects. An analysis of the blood (BL) on Ouchterlony plates is shown in Figure 2. When titered, two lines of precipitation were still visible at a dilution of 1:16. The second plate shows two and possibly three lines of precipitation. Two of these show some similarity to that of PA. None shows any similarity to that of either EF or LF.

* Unpublished observations.

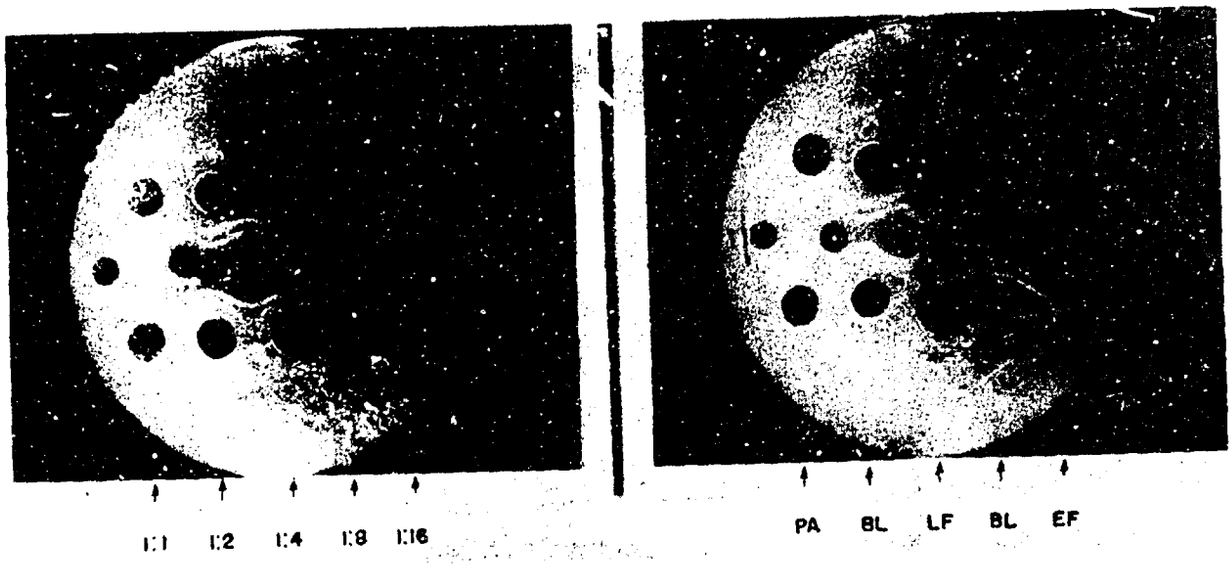

Figure 2. Serological Examination of Monkey Blood after Death from Anthrax.

The second monkey had been challenged with V1b spores and died without further treatment. Within 30 minutes after death, 1.0 ml of the blood killed a rat in 33 minutes; 2.0 ml killed in 5 minutes. After storage at 4 C for 24 hours, 0.5 ml killed in 103 minutes, 1.0 ml killed in 44 minutes, and 2.0 ml killed in 13 minutes. Ouchterlony analysis showed one line with a titer of 1:8. This line showed a line of identity with PA. The presence or absence of LF could not be determined clearly.

In a third monkey, whose serum was examined for the presence of the three components of toxin from the Sterne strain, evidence was found for the presence of all three components of the Sterne strain toxin as well as of some additional unidentified components.

D. EVIDENCE FOR DISSOCIATION OF THE TOXIN MOLECULE

The terminal serum of six monkeys injected with 1×10^8 V1b spores was collected and assayed (Table 3). The sera were then frozen and stored at -20 C for 3 days. After thawing, they were again assayed. Table 3 showed a marked loss in activity as measured by rat lethality and guinea pig skin edema. The Ouchterlony titer did not decrease and, in fact, the number of lines increased. These results indicate dissociation of a complex with concomitant increase in serological activity and decrease in biological activity rather than destruction of a single molecule. Similar results were found with the serum from monkeys that died following challenge with strain 116 spores.

In another type of experiment, the terminal blood was stored at 4 C and assayed periodically. The rat lethality rose to a maximum at 6 to 12 hours after death and then decreased. The maximal titer and number of lines by Ouchterlony assay paralleled the maximal rat lethality. These results indicate that molecular changes occur even at 4 C.

TABLE 3. EFFECT OF FREEZING AND THAWING ON ANTHRAX TOXIN PRODUCED IN VIVO

Animal Number	Fresh Serum				Frozen and Thawed Serum					
	Ouchterlony[a]		Guinea Pig[b]	Rat,[c] min	Ouchterlony[a]				Guinea Pig[b]	Rat,[c] min
	2 Lines	1 Line			4 Lines	3 Lines	2 Lines	1 Line		
1	Lost		1:80	75	-	1:2	1:16	1:16	NE[d]	1,110
2	-	>1:32	1:80	61	-	-	1:16	1:16	NE	75
3	-	1:16	1:20	50	-	-	-	1:32	NE	73
4	1:4	1:16	1:20	50	1:4	1:8	>1:32	-	NE	113
5	-	1:8	1:80	101	-	1:16	-	-	NE	Survived
6	-	>1:32	1:80	63	-	>1:32	-	-	NE	Not done

a. Highest titer at which indicated number of lines is visible.
b. Highest titer that still gives visible edema.
c. Average time to death of two rats following IV injection.
d. NE = no edema.

IV. DISCUSSION

Our observations with both guinea pigs and monkeys support those of Smith et al.[4] that toxin is found in the thoracic and peritoneal fluid at death. The presence of toxin in the lymph and serum of monkeys infected with anthrax has been reported.[13] At least two distinct antigens were visualized in the thoracic and peritoneal fluid and in the sera of both guinea pigs and monkeys dying of anthrax.

When rats were injected with the serum or thoracic or peritoneal fluid they died in the same manner as did rats that had received anthrax toxin. Although Sargeant et al.[6] did find some minor differences in the pattern of lines produced on Ouchterlony plates by toxin isolated from in vitro vs. in vivo sources, they concluded that the major components appeared to be the same. Fish et al.[7,14] reported that they were able to identify only two lines of precipitation in in vitro - produced toxin. Therefore, the toxin produced in vivo under these conditions appears to resemble that produced in vitro.

The results reported here also indicate that the same sort of antigens are produced by V1b, 116, and 770 strains as are produced by the Sterne strain.

A kinetic study of the formation of toxin indicates that it can first be visualized about 13 to 15 hours prior to death in the guinea pig and 6 hours prior to death in the monkey. These results, and those in which the correlation between number of organisms and amount of toxin present in the blood was determined, agree precisely with the observations of Smith et al.,[1] Tempest and Smith,[5] Lincoln et al.,[15] and Mahlandt et al.[16]

The toxin in the serum of the guinea pig between 8 to 13 hours prior to death exhibited only one strong line of precipitation by the Ouchterlony technique. Between 2 and 7 hours prior to death a second line of precipitation became visible; later than this a third line became visible. These results indicate either that the formation of the different toxin components in vivo, as demonstrated by their appearance in the blood stream, is different or that the toxin is synthesized as one multicomponent aggregate that is subsequently broken apart.

Although antisera can be formed by repeated injections of either the partially purified toxin components or spore suspensions, the specificity and extent of the response have not been satisfactory. Sargeant et al.[6] called attention to the fact that none of the purified preparations they examined was serologically homologous. They especially noted that some preparations of EF contained nonimmunogenic but serologically active PA. Our results indicate that either the antigens we used were not homologous or that common antigenic sites existed, because PA and EF led to the formation of antibody against PA but LF led to the formation of antibody against both PA and LF.

Both the toxin components and the spore suspensions appear to be poor antigens. Repeated injections are needed before any titer is observed; the antibody titer soon returned to zero after immunization ceased. The best titer was obtained with the more virulent organisms. It is probable that the animal had a low-grade infection that kept the concentration of antigen higher for a longer period of time. One of the more interesting observations from this work, though, is that often the animals have a fairly high degree of protection (immunization) with no visible antibody titer.

Both our experiments and those of Belton and Henderson[17] have shown that monkeys are able to build an antibody titer rapidly following rechallenge. We have verified the observation of Ward et al.[18] that immunized animals die with both toxin and antibody present in their blood.

Contrary to the observations of Watson et al.[19] and Ward et al.[18] we do not find any alteration in the pattern of serum electrophoresis at death.

In several instances, in vivo - produced toxin killed a rat in an extremely short time. The Ouchterlony titer was not very high and, in fact, we could not even detect LF in the serum, yet the rat died. We are tempted to believe that this is the result of a particular configuration of the toxin molecule that is readily altered outside the body.

The evidence in the literature that the toxin molecule does not have a stable configuration or composition is quite prevalent.[2-4,14,20-26] In every case, those workers found that the molecule could dissociate or aggregate to yield a compound with slightly altered properties.

The fact that the Ouchterlony readings, guinea pig skin edema, and rat lethality titers of monkey serum from animals dying of anthrax changed so markedly after storage at -20 C is further evidence for the lability of a special toxin configuration. The results from the experiments on the kinetics of toxin formation in the guinea pig during the disease indicate that either (i) the rate of formation or release into the blood stream of the various toxin components is different or (ii) the toxin complex is dissociated into several components in vivo. This dissociation affects the properties of the toxin to a different extent depending upon the parameter used to measure activity.

The toxin not only loses its biological activity after freezing and thawing but yields an increase in the number of lines of precipitation by Ouchterlony analysis with no decrease in the terminal titer. This can most easily be explained by dissociation of a complex molecule or aggregate rather than by destruction of a single substance. It is important to remember that the results reported here were obtained with crude toxin and that the purified toxin or its components may behave differently.

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13. ABSTRACT

Specific anthrax antigens were demonstrated in the blood of animals dying from anthrax. These antigens appeared in the blood when organisms were first detected and their concentration continued to increase as the concentration of organisms increased. These antigens did not elicit a strong antibody response. In some respects, the toxin produced in vivo is different from that produced in vitro (may kill more rapidly, harder to detect lethal factor). The in vivo toxin also exists as an aggregate whose biological and serological activity depends upon its particular composition and/or configuration.

14. Key Words

*In vivo	Dissociation
*Anthrax	Bacillus anthracis
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Blood	Assay
Kinetics	Animals
Antiserums	*Toxins

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